



Mouse cPLA2 γ , a novel oocyte and early embryo-abundant phospholipase A2 gamma-like protein, is targeted to the nuclear envelope during germinal vesicle breakdown

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Received for publication 5 September 2004, revised 17 March 2005, accepted 22 March 2005

Available online 30 April 2005

Abstract

This report documents the characterization of a novel mouse oocyte protein which was originally identified by microsequence analysis of a 67.8 kDa protein spot (pI 5.7) on a Coomassie-stained two-dimensional (2D) gel of murine egg proteins. Tandem mass spectroscopic analysis of the peptides obtained from the cored protein yielded sequences that appeared to match only ovary, egg, and preimplantation embryo cDNAs. We then cloned the novel gene by RACE-PCR, and analysis of the deduced cDNA sequence found that this maternal product was ~56% identical to human cytosolic phospholipase A2 γ (cPLA2 γ). Based on this sequence homology, we named the molecule mouse cytosolic phospholipase A2 γ (cPLA2 γ). As with human cPLA2 γ , mouse cPLA2 γ contains a lipase consensus sequence and lacks the calcium binding domain that is found in other PLA2 proteins. However, mouse cPLA2 γ is different from human cPLA2 γ in that mouse cPLA2 γ expression is restricted to the ovary and that the protein does not contain the myristoylation and prenylation lipid-anchoring motifs that are present in human cPLA2 γ . Within oocytes, mouse cPLA2 γ localizes mainly to the oocyte cortex and to the nucleoplasm. Interestingly, during germinal vesicle breakdown, mouse cPLA2 γ aggregates dynamically relocate from the oocyte cortex to the nuclear envelope, suggesting a possible role for this putative egg-restricted phospholipase A2 γ in membrane remodeling. Furthermore, mouse cPLA2 γ protein continues to be expressed in the embryo until the 4–8-cell stage of development, suggesting that mouse cPLA2 γ may function as a previously uncharacterized maternal effect gene.

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Keywords: cPLA2 γ ; Maturation; Fertilization; Preimplantation

Introduction

Oocyte maturation, fertilization, and preimplantation development are facilitated by maternal gene products which accumulate in the oocyte during its growth (Latham and Schultz, 2001). In an effort to further elucidate the molecular identities of these poorly characterized molecules, we have cloned and characterized a subset of genes from the mouse oocyte proteome that encode highly abundant and

oocyte-restricted proteins. Bioinformatic analysis of one such gene product identified a novel egg protein, which we named mouse cytosolic phospholipase A2 γ (cPLA2 γ) based on its ~56% identity to the previously characterized human cPLA2 γ enzyme (Underwood et al., 1998). Phospholipase A2 enzymes catalyze the hydrolysis of membrane glycerophospholipids at the *sn*-2 position producing free fatty acids and lysophospholipids (Brown et al., 2003). If arachidonic acid is the esterified fatty acid, then downstream metabolic enzymes, such as cyclooxygenases, convert this fatty acid to various bioactive lipophilic compounds called eicosanoids, which include prostaglandins (PGs) and leukotrienes (LTs) (Lucas and Dennis, 2004). Primarily,

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three different kinds of phospholipase A2's have been identified and cloned in mammals: calcium-independent cytosolic phospholipase A2 (iPLA2), secretory phospholipase A2 (sPLA2), and cytosolic phospholipase A2 (cPLA2) (Kudo and Murakami, 2002). The Ca^{2+} independent PLA2 (iPLA2) family contains two enzymes, VIA (iPLA2 β) and VIB (iPLA2 γ), which may play a major role in phospholipid remodeling (Akiba and Sato, 2004). The secretory PLA2 family consists of low-molecular weight Ca^{2+} -dependent secretory enzymes that have been implicated in a number of biological processes, such as regulation of eicosanoid production, inflammation, and host defense (Kudo and Murakami, 2002). The cytosolic phospholipase A2 (cPLA2) family, known as Group IV according to the new nomenclature, consists of three isozymes, IVA (cPLA α), IVB (cPLA β), and IVC (cPLA γ), with molecular masses of 85, 110, and 60 kDa respectively (Kudo and Murakami, 2002). The most studied member of this group is cPLA α , which contains two domains, a calcium-dependent lipid domain (CaLB) and a catalytic domain, but lacks any detectable sequence homology with the secreted forms of PLA2 (Clark et al., 1991). cPLA2 γ , the least studied member of this group, shows calcium-independent catalytic activity and is constitutively associated with the lipid bilayer via a prenylation moiety located at the C-terminus. This prenylation site is critical for membrane localization and thus for cPLA2 γ function (Murakami et al., 2003; Underwood et al., 1998).

While there is considerable evidence in the literature suggesting that PLA2s are important for several stages of early embryonic development (Carnero and Lacal, 1993; Farber et al., 1999; Riffo and Parraga, 1997), the molecular identities of these phospholipases have not been previously documented. Here, we report the characterization of mouse cPLA2 γ , an egg and embryo-abundant phospholipase A2 γ that localizes mainly to the cortex and nucleoplasm of oocytes and early cleavage division blastomeres. Given its homology to phospholipase A2, we predict that mouse cPLA2 γ may be involved in lipid metabolism and membrane remodeling events during oogenesis and preimplantation development. Our finding that mouse cPLA2 γ concentrates at the nuclear envelope during germinal vesicle breakdown supports this prediction.

Materials and methods

Identification of mouse cPLA2 γ peptide sequences by tandem mass spectroscopy

Mouse oocyte proteins (2850) were collected and separated on 16 cm 2D electrophoretic gels, as previously described (Wright et al., 2003). A protein spot of approximately 67.8 kDa (pI 5.7) was cored from a Coomassie-stained gel, digested with trypsin, and micro-sequenced by tandem mass spectrometry as previously

described (Wright et al., 2003). The obtained peptide sequence information was then compared against database sequences using the NCBI Blast Website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

RACE-PCR cloning of mouse cPLA2 γ cDNA

The mouse cPLA2 γ open reading frame was amplified from a mouse oocyte adapter-ligated cDNA library by RT-PCR using DNA polymerase (Amplitaq Gold, Perkin-Elmer, Norwalk, CT) as previously described (Wright et al., 2003). Cycling parameters were: 94°C, 10 min; 94°C, 15 s; 60°C, 30 s; 72°C, 2 min for 40 cycles; and 72°C, 10 min. PCR of plasmid templates was performed under the same conditions except that Taq polymerase (Promega Advantage 2) was used. The primers for cloning the full length ORF of the mouse PLA2 γ into the TOPO cloning vector (Invitrogen) were 5'-ATG-GAACTAAGCTCTGGGGTTC-3' and 5'-AGGGTGTG-TATAGATTCCTA-3', and the AP1 primer was supplied within the Marathon ready cDNA kit (Clontech, Palo Alto, CA).

Northern blot analysis

A randomly primed probe was generated corresponding to the 900 bp N-terminal region of the mouse cPLA2 γ using the Prime-a-Gene Labeling System (Promega, Madison, WI). Northern blot analysis was performed as described previously (Wright et al., 2003). Signals were detected by exposure to X-ray film for 10 days.

Expression of recombinant protein and antibody production

Primers were designed to generate a PCR product encoding amino acids 1–110 of the mouse cPLA2 γ open reading frame. The sense gene-specific primer sequence was 5'-ATGGAAGCTCTGGGGTTC-3' with the three prime end containing an engineered *Bam*HI restriction site. The antisense gene specific primer sequence was 5'-TCCAGTCTAAAGAAGCAAGTG-3' and contained an engineered *Xho*I restriction site. The primers were used to amplify the cDNA fragment, and the PCR product was cloned into the *Bam*HI–*Xho*I restriction sites of the pET22b expression vector (Novagen, Madison, WI). The recombinant protein was then expressed and purified as described previously (Wright et al., 2003). Preimmune serum was collected from adult male guinea pigs, and each animal was then injected with 100 μ g of purified recombinant mouse cPLA2 γ in an emulsion with Freund's complete adjuvant. The animals received one booster immunization with 50 μ g of the purified recombinant mouse cPLA2 γ in Freund's incomplete adjuvant at 3-week intervals. The polyclonal sera was then collected and prepared as previously described (Wright et al., 2003).

Western blot analysis of mouse cPLA2 γ in oocytes and eggs

We performed 1 and 2D Western blot analysis to confirm the specificity of the mouse cPLA2 γ antibody. For 1D analysis, BL21 cells expressing recombinant cPLA2 γ were sonicated and 20 μ g of bacterial protein as well as fifty oocytes were solubilized in Laemmli buffer (Laemmli, 1970) and heat denatured at 95°C. The samples were then loaded onto a 12.5% linear gel and separated at 100 V for 3 h. Proteins were then electro-transferred onto 0.2 μ m nitrocellulose (Bio-Rad Laboratories, Hercules, CA) for 40 min at 100 V for Western blotting. For 2D Western blot analysis, proteins from ~800 zona-intact ovulated metaphase-II-arrested eggs were extracted, separated by 2D gel electrophoresis (16 \times 16 cm PAGE), and immunoblotted as previously described (Wright et al., 2003). A 1:2000 dilution of the mouse cPLA2 γ preimmune and immune antisera were used for the 1 and 2D Western blot experiments.

For analysis of mouse cPLA2 γ protein expression during oocyte maturation and preimplantation development, germinal vesicle oocytes were obtained as described previously (Coonrod et al., 1999, 2001). Metaphase II eggs (MII) were isolated from the oviducts of superovulated female mice. Pronuclear zygotes (PN), 2-cell, 4–8-cell, morula (Mo), and blastocyst (Bl) embryos were isolated from the oviducts and uterus of superovulated and mated female mice. Twenty oocytes/embryos from each stage were boiled for 5 min in Laemmli buffer and directly loaded into a 10% SDS-PAGE gel. Proteins were separated at 200 V for 50 min and then transferred to a nitrocellulose membrane by applying a current of 90 V for 90 min. All blots were blocked with 5% nonfat dry milk in TBS with 0.1% Tween-20 (TBS-T) for 30 min, washed, and incubated with a 1:5000 dilution of anti-recombinant PLA2 γ guinea pig IgG. The blots were then washed 3 times for 10 min in TBS-T and incubated with a 1:10,000 dilution of peroxidase-conjugated goat anti-guinea pig IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h. Following incubation in secondary antibody, the membranes were washed 3 times for 10 min in TBS-T, and ECL reagent (Amersham Corp. Buckinghamshire, UK) was applied for 2 min and developed as previously described (Coonrod et al., 1999). The experiments were repeated three times.

RT-PCR of mouse cPLA2 γ in oocytes and eggs

For analysis of RNA expression of mouse cPLA2 γ during maturation and preimplantation development, germinal vesicle oocytes were obtained as described previously (Coonrod et al., 1999, 2001). Metaphase II eggs (MII) were isolated from the oviducts of superovulated female mice. Pronuclear zygotes (PN), 2-cell, 4–8-cell, morula (Mo), and blastocyst (Bl) embryos were isolated from the oviducts and uterus of superovulated and mated female mice. Total RNA from 20 oocyte/embryos at each stage was extracted using

200 μ l of TRIzol reagent as described in manufacturer's protocol. RT-PCR was performed using a one-step RT-PCR kit (Sigma Pro HSRT-100) under the following experimental conditions: 2 μ l of total RNA from each stage was added to 25 μ l reactions containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 μ M dNTPs, 0.4U/ μ l RNase inhibitor, 0.4 μ M of each primer, 0.4U/ μ l Avian Reverse Transcriptase, and 0.05U/ μ l JumpStart AccuTaq LA DNA polymerase. The following mouse cPLA2 γ ortholog primers were used: 5'-GGTAAGGACTGCTGACAGTGGCTAGCT-3'; 5'-CCTCCAGAAGTGTCAAGCTGGTAGATGT-3'. Thermocycler settings consisted of 30 min at 50°C, 2 min at 95°C, and 34 cycles at 95°C (15 s), 52°C (30 s), and 72°C (1 min) followed by 10 min at 72°C. Twenty percent of product was later analyzed on a 1.4% agarose gel. The experiments were repeated three independent times.

Immunohistochemistry of ovarian sections

Adult female mouse ovaries were fixed in 4% paraformaldehyde, embedded in paraffin wax, cut in 5 μ m sections, and processed for immunohistochemistry. In brief, the paraffin wax was removed using Neo Clear Clarification™ (EM Science, NJ, USA), and the section was rehydrated through a graded series of ethanol solutions (100–70%) in H₂O. To localize the mouse cPLA2 γ in the histological sections, endogenous peroxidase activity was first removed by treatment with 3% H₂O₂ for 20 min at room temperature. After washing with PBS-BSA (0.15%), slides were blocked for 20 min with BSA (2%) and then incubated overnight with the mouse cPLA2 γ guinea pig preimmune or immune antisera (1:500 in PBS-BSA 0.15%). Next, the slides were washed and incubated with biotinylated secondary antibody for 30 min, washed again, and treated with VECTASTAIN elite ABC reagent (Vector Laboratories, Burlingame, CA) for 30 min. Finally, the sections were incubated with a peroxidase substrate solution until the desired staining levels were obtained, rinsed in tap water, counterstained with hematoxylin, cleared with Neo Clear Clarification™, mounted on slides using mounting media (Permount, Fisher Chemicals), and visualized at 100 \times .

Indirect immunofluorescence of oocytes and embryos

All oocytes and embryos were obtained from ICR 25–30 g females. Germinal vesicle oocytes were obtained as described previously (Coonrod et al., 1999, 2001). Metaphase II eggs (MII) were isolated from the oviducts of superovulated female mice. Pronuclear zygotes (PN), 2-cell, 4–8-cell, morula (Mo), and blastocyst (Bl) embryos were isolated from the oviducts and uterus of superovulated and mated female mice and immediately fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Following fixation, oocytes and embryos were washed 5 times in IF media (PBS + 1% BSA + 0.5 NGS) and then permeabilized with 0.5% Triton-X 100 in PBS for 30 min.

Oocytes and embryos were then washed 5 times and incubated with the mouse cPLA2 γ guinea pig preimmune and immune antisera 1:300 in IF media for 1 h at room temperature. Oocytes and embryos were washed 5 times and incubated for 3 h at room temperature with goat anti-guinea pig FITC-labeled secondary antibody (Jackson Immuno-research). Experiments were repeated at least three times, using 20 to 30 oocytes/embryo per developmental stage, pooled from 3 mice.

To evaluate GVBD, geminal vesicle oocytes were collected and either immediately fixed in 4% paraformaldehyde in PBS (time 0) or incubated at 37°C and 5% CO₂ in maturation buffer (TYH buffer) as previously described (Wright et al., 2003). The oocytes were then fixed at 40, 60, 90, 120 min, and 24 h, permeabilized, and washed as described above. The oocytes were then incubated with the mouse cPLA2 γ guinea pig preimmune and immune antisera 1:300 in IF media for 1 h at room temperature followed by an incubation with a 1:1000 dilution of anti-lamin A/C IgG (Santa Cruz, CA) or with a 1:2000 dilution of anti- γ -tubulin (Sigma, MO) at 4°C overnight. The oocytes were washed 5 times and incubated for 3 h at room temperature with goat anti-guinea pig FITC-labeled secondary antibody (Jackson Immuno-research) together with goat anti-rabbit Texas Red-labeled secondary antibody (Jackson Immuno-research) to recognize lamin A/C or γ -tubulin and Hoechst to stain the DNA. Finally, oocytes/embryos were mounted on slides and visualized at 1000 \times under a Zeiss Axiovert-200 deconvolution fluorescence microscope and imaged. Experiments were repeated at least three times, using 30 to 40 oocytes per time point which were pooled from 4 to 6 mice.

Results

Tandem mass spectroscopic identification of mouse cPLA2 γ

Protein extract from 2850 zona-free mouse eggs was separated by two-dimensional electrophoresis and stained with Coomassie (Fig. 1). One protein spot, later designated as mouse cPLA2 γ (Fig. 1, arrow), with a molecular weight of 67.8 kDa and isoelectric point of 5.7, was cored from the gel, digested with trypsin, and microsequenced by CAD mass spectrometry. This analysis revealed 21 unique peptides that did not match previously characterized protein sequences in the nonredundant database. We therefore concluded that this protein was novel. However, one peptide with the sequence FIEGPVTYSEAPR did match an EST clone, C88186, from a fertilized egg cDNA library.

Cloning and characterization of mouse cPLA2 γ

Oligonucleotide primers (designed from either the FIEGPVTYSEAPR peptide sequence or from the matching EST sequences) were used for repeated RACE-PCR reactions to amplify a 2701 bp cDNA from the ovarian

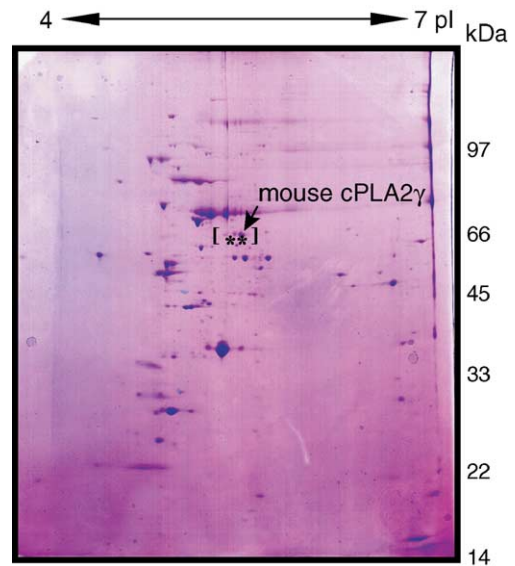


Fig. 1. Location of mouse cPLA2 γ (67.8 kDa, pI 5.7) on a Coomassie-stained two-dimensional electrophoretic gel of 2850 extracted mouse oocyte proteins. The arrow indicates a spot that was cored from the gel for tandem mass spectroscopic identification of the mouse cPLA2 γ peptides. Two other protein spots of higher acidity (to the left of the arrow and contained within brackets) were later shown to be reactive with the mouse cPLA2 γ immune sera, possibly indicating post-translationally modified isoforms.

cDNA library which contained the mouse cPLA2 γ open reading frame. The cPLA2 γ cDNA (GenBank accession number AY694793) was inserted into a cloning vector, and subsequent sequence analysis revealed a 75 bp 5' untranslated region, a contiguous open reading frame of 1791 bp (encoding a deduced protein of 597 amino acids), and a 796 bp 3' untranslated region (Fig. 2). The untranslated flanking regions are shown in lower case letters in Fig. 2. The computed mass and isoelectric point (ExPASy compute pI/MW algorithm) of the deduced amino acid sequence are 66 kDa and 5.36 pI, respectively, which closely match the mass and pI of the protein spot that was cored from the 2D gel for TMS analysis. Thirteen of the 21 peptides originally identified by TMS were found in the deduced amino acid sequence and are underlined in Fig. 2. Comparing deduced amino acid sequence against the database sequence using the NCBI conserved domain function (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) resulted in significant similarities with both a cytoplasmic phospholipase A2 (PLA2) domain (E value = $2e - 31$) and a PLA2 B lysophospholipase catalytic domain (E value = $3e - 51$). The first domain is found in enzymes that hydrolyze arachidonyl phospholipids and overlaps the ~ 300 aa N-terminus of the mouse cPLA2 γ . The latter, observed between residues 45 and 503, is found in enzymes that catalyze the release of fatty acids from lysophospholipids.

Comparison of the sequence against the NCBI non-redundant database using the BLASTp algorithm found 100% identity with a deduced protein sequence (XP 149881.2) named phospholipase A2 gamma, group IVC (cytosolic calcium-independent). The sequence is $\sim 80\%$

gactcactatagggctcagcgccgcccggcgaggtatggctctcgtgtgctcctgct
 cctgcacatgcttgagttccagctcagctccttggtgataaacacagcagcatggaacta
 M E L
 AGCTCTGGGGTCTGCCCTGCCACTAGACTCCAGGAAGCAGAAAGGCAGCTGTGCACAAA
 S S G V C P A T R L Q E A E K A A V H K
 CGCAGTCCCAAGGTTTGGAGGCTCTACGAAAGCTCAACATCCAAGCTGACCGGCTCCCA
 R S P K V L E A L R K L N I Q A D Q A P
 GTCATTGCTGCTTGGGCTCTGGCGGGGGGCTCGGGGCCACATCGCTTGTCTTGGTGTG
 V I A V L G S G G G L R A H I A C L G V
 CTGAGTGAGCTGAAAGAACTTGGCCTGTTGGATGCTGTACATACCTCGCAGGGGCTCTCT
 L S E L K E L G L L D A V T Y L A G V S
 GGGTCCACTTGGGCACTGCTTCACTCTACCAAGAAATGGAATGGAAGGATAGAA
 G S T W A L S S L Y T K N G N M E G I E
 GAGGAGCTGAACATCGGTATGAGAAGATGAGTGGGACTTTCATGAGAGCCTGGAGAAA
 E E L K H R Y E K N E W D F H E S L E K
 GCCATCCAGGCTCAAGAGGAGGAGATCTCCCTGACTGACTTTTGGGCTATTTAAT
 A I Q A S K R E N Y S L T D F W A Y L I
 GTTTCAGGCAAACTCAGAGAAGTTCAGGATTCGAATTTGCCAGTCTAAGAAAGCAAGTG
 V S R Q I R E L Q D S N L S S L K K Q V
 GAGAAGGAGTGTGCTGCCCTATCCATCTTTCAGCCATGATGAGGAGCTTCTGGCTGAT
 E E G V L P Y P I F A A I D E D L A D
 TGGAGGAGAGGAAAACCTCAGAATTCCTGGTTGAATTCACCTCCATCATGCTGGCTAC
 W R E R K T Q N S W F E F T P H H A G Y
 CCTGCATCTGGGCTTATGTCCTCCATCAGAGATTTGGAAGCAGATTTGAGAATGGGAAA
 P A L G A Y V P I T E F G S R F E S L E K
 CTGGTTAAATCTGAGCCTGAGAGAGATGACTTCTCTGAGAGGTTTATGGGGAAGTGT
 L V K S E P E R D L T F L R G L W G S A
 TTTGCTGATTTAAAGAAATTAAGAATTATATTTTGAACACTCTCAGGAACCCCTTTGGA
 F A D I K E I K N Y I L N Y F R N P F G
 AAATTGAAGTTTATAGAAGGACCACTGACATCTCAGAAGCACCAAGGATGAATGTGGAT
 K L K F I E G P V T Y S E A P R M N V D
 GCAATGCTCTTGGATTTAGTGATGGCTTATTTACAGATATGAATGACCCAGCATCAAG
 A M L L D L V M A Y F T D M N D P S I K
 GATAAGCTCTGTGCCCTTCAGCAGGCTCTGGGTACTGAGACAGATGAATTTGGCATAGAG
 D K L C A L Q Q A L G T E T D E F G I E
 ATGGCTGAGATCATCCGAATTTGGAATGAGACCTCCGACAGAGAAGAGCAGTTTCTC
 M A E I I Q N W N E T S A E K K E Q F L
 GACCATCTGTTGATCGCTTCAAGAGACATCAGGAGACACCAACATACAGTTTGTATG
 D H L L D R F K K T Q E D T T T Y S L M
 AACTGGAACACGGGTCTAGTTTGGGACCGTTGCGTTTGTGAATGAAACTCGCAAGTGT
 N W N T G L V W D R C V F V N E T R K C
 GTCTCCAAATGGGAGTGGGGAAGTCTTTACAACCTCCTCTATAAACACGGTAAATTTGCA
 V S K W Q W G T V T Y N F L Y K H G K I A
 GATGAGACCATGTGCGAGCGAGAGCTTCTCCATCTGGTGGATGCTGGTTTGGCATCAAC
 D E T M C S R E L L H L V D A G F A I N
 ACTCCCTATCCACTTGTCTGCTCTGTGCGTGAAACTCACCTCATCTCTCATTGAC
 T P Y P L V L P P V R E T H L I L S F D
 TTCAGTCTGGGAGCCCATAGAGACCATCAGGGCACAGCAGACTCTGCCAACGCCAT
 F S A G D P L E T I R A T A D Y C Q R H
 GAAATCCCTTTCTGAGTGAGCGAGGATGAGTGAAGGAATGGGCCAAAGCCCAAGCA
 E I P F F E V S E D Q L K E W A K A P A
 AGCTGCTATGCTCTCAGAGGGGAAACAGCCTGTTGTGATGCACTTTACTCTGTTCAAC
 S C Y V L R G E T G P V V M H F T L F N
 AAAGAACACTGTGGAGATGATTTGAGACATGGAGAAATATGGGACAGTAAACTA
 K D N C G D D I E T W R K K Y G T V K L
 TCTGACTCATACACACAGACCTGGTGAGAGATTGCTGAGGGTATCCAAGGAGAAATGTG
 S D S Y T P D L V R D L L R V S K E N V
 AAGAAAACAAAATTAATCTCTCAGTGAGATGAGAAAGTGGCTGGGAAATCCTGGGAAAC
 K K N K I N I L S E M R K V A G N P G N
 ATCCCAAGAGTGAACAAGAGGCTGCTTGGGAGACAGAGTAAAGATPCCCAAGGCTCT
 I P R V N K E A L D R V N K D P Q G S
 CAGACTGTGGAGTTAAGAAATCCCAACATATCTAAGGATTAAGgattcctttgcaacta
 Q T V E F K K S H N I S K D *
 cctggagagttggcatctattagtggaactcaccacgtgtctctcctcaaaagtcaccctg
 aagctgtctctttgtgaagaagaactgcagttttcagggtactgtgggctactgcttca
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 tagctattaccattgagcaagtctccagagacctagatggtgtctttgatagcattatt
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 acatgtctgttatatgctgtctatctgtgccttagtgctccttctgttgcctact
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 tcatgttaatacacaggcaagttccttcacatgaattccttttaatacatctatggaataa
 atgtgcatcttggtgtgctcctggtcttttcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Fig. 2. Complementary DNA sequence and deduced amino acid sequence of mouse cPLA2 γ . The untranslated flanking regions are shown in lower case letters. Peptide sequences originally obtained by mass spectrometry are underlined.

identical to rat PLA2 γ (a deduced sequence), ~56% identical to previously characterized human cPLA2 γ , and ~27% identical to chicken cPLA2 α . A Blast analysis of the mouse cPLA2 γ nucleotide sequence against the

genome reveals that mouse cPLA2 γ is located on mouse chromosome 7 and is flanked by two genes, SLC27A5 and APOC1, which are also associated with lipid metabolism. Analysis of human cPLA2 γ finds that this gene is located on chromosome 19 at position q13.3. This position also contains the human orthologs of mouse SLC27A5 and APOC1, thus suggesting that mouse and human cPLA2 γ are, in fact, orthologous. A further comparison of the mouse cPLA2 γ sequence with human cPLA2 γ shows that both molecules lack the cPLA2 α calcium-dependent lipid binding domain yet retain the residues which comprise the cPLA2 α catalytic triad (Fig. 3, arrowheads). However, as opposed to human cPLA2 γ , mouse cPLA2 γ lacks obvious N-terminal myristoylation (M-G-X-X(S/small uncharged)-X) and C-terminal prenylation (-CCLA) membrane anchoring motifs (Fig. 3, boxes), indicating that perhaps mouse cPLA2 γ is not membrane anchored.

Mouse cPLA2 γ expression is limited to the ovary

An EST database search using the mouse cPLA2 γ ORF nucleotide sequence found multiple identical or near identical matches with ovary, unfertilized egg, fertilized egg, 2-cell, and 4–8-cell cDNAs. Single matches were also obtained with cDNA clones derived from adult male colon (GenBank accession no. BB624941) and infiltrating ductal carcinoma (GenBank accession no. BX524494) somatic tissues. This information led to the prediction that mouse cPLA2 γ may either be an egg-abundant or egg-specific transcript whose expression persists throughout the early stages of cleavage. Mouse cPLA2 γ expression was then examined in various tissues using Northern blot analysis (Fig. 4). Two blots, one containing 5 μ g multi-tissue poly-A⁺ RNA, and the other, 40 μ g of total ovarian RNA, were probed with a ³²P-labeled random-primed 900 bp mouse cPLA2 γ N-terminal DNA fragment. The probe, while not binding to RNA from somatic and testicular RNA, did recognize a ~2.7 kb ovarian transcript (Fig. 4). Subsequent multiple tissue Northern blotting experiments using a single membrane showed similar results (data not shown).

A 67.8 kDa (pI 5.7) protein spot which contains mouse cPLA2 γ peptide sequence is specifically recognized by anti-mouse cPLA2 γ antibodies

cDNA encoding an N-terminal ~110 amino acid region of mouse cPLA2 γ was cloned into a bacterial expression vector, expressed, purified, and used as an immunogen for mouse cPLA2 γ antisera production in guinea pigs. Antibody specificity was first confirmed by 1D Western blotting (data not shown) which found that the mouse cPLA2 γ antisera was reactive with recombinant cPLA2 γ and with an appropriately sized egg protein. On 2D immunoblots, the anti-mouse cPLA2 γ sera reacted with a ~67.8 kDa protein (pI 5.7), which is the precise location of the protein spot that was originally cored from the Coomassie-stained gel of

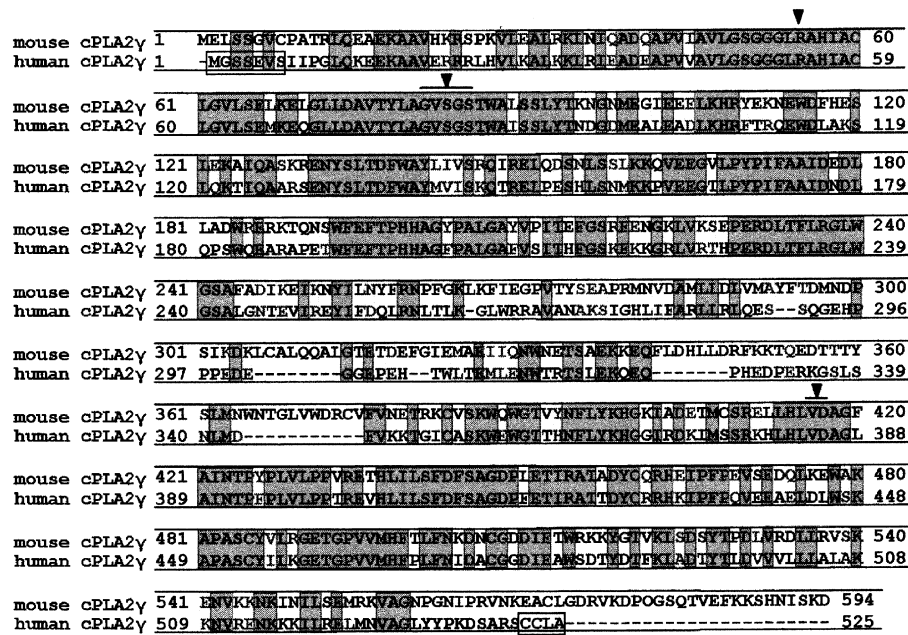


Fig. 3. Amino acid sequence comparison of mouse cPLA2γ with human cPLA2γ. The sequences were aligned using the ClustalW alignment program in BioEdit sequence alignment editor. Identical residues are shown in dark gray boxes, and conserved residues are shown in light gray boxes. Dashes indicate gaps inserted to maximize sequence alignment. As opposed to human cPLA2γ, mouse cPLA2γ lacks obvious N-terminal myristoylation and C-terminal prenylation membrane anchoring motifs (boxes). Catalytic triad is shown with arrowheads.

mouse oocyte extracts for microsequence analysis (Fig. 5, arrow). The preimmune sera was not reactive with egg proteins (Fig. 5). These results provided immunological proof that specific antibodies had been generated against the original cored protein. Interestingly, the mouse cPLA2γ immune sera was also reactive with two other slightly more acidic protein spots of similar molecular weight (asterisk in Figs. 1 and 5), indicating that mouse cPLA2γ may be post-translationally modified.

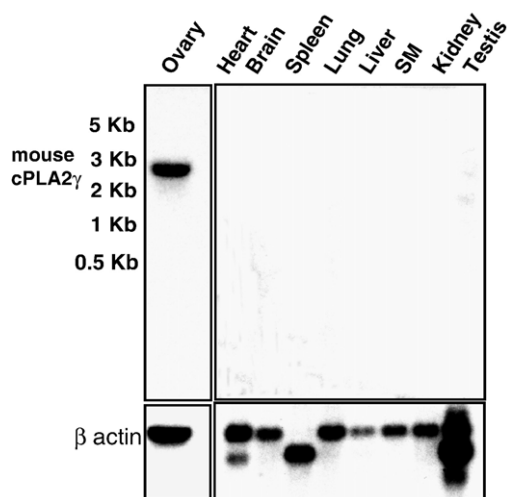


Fig. 4. Mouse cPLA2γ tissue distribution. Northern blot analysis was performed on various tissues using a P³²-labeled 900 bp mouse cPLA2γ DNA fragment. Mouse cPLA2γ was detected as a single ~2.7 kilobase band in the ovarian RNA lane. A β-actin probe was used to control for RNA loading. Tissues included ovary, heart, brain, spleen, lung, liver, skeletal muscle (SM), kidney, and testis.

Developmental expression and subcellular localization of mouse cPLA2γ

Immunohistochemical localization of mouse cPLA2γ in adult ovaries showed that the protein localized mainly to oocytes in preantral and antral stage follicles, as indicated by the strong brown staining (Fig. 6). Weak staining of surrounding stromal tissue was also observed. While the preimmune sera was not reactive with oocytes, it was weakly reactive with stromal tissue indicating that the staining of the stromal cells with the immune sera was likely non-specific (Fig. 6, inset). No detectable staining was seen in adjacent sections when the primary antibody was omitted (data not shown).

We next investigated the expression and subcellular localization of mouse cPLA2γ protein during oocyte maturation, fertilization, and early embryonic development. Immature germinal vesicle stage oocytes (GV), mature metaphase-II-arrested eggs (MII), pronuclear zygotes (PN), 2-cells, 4–8-cells, morulas (Mo), and blastocysts (BI) were collected in vivo and immediately frozen for RT-PCR and Western blot analysis or fixed for immunofluorescence staining.

RT-PCR analysis showed that mouse cPLA2γ mRNA was expressed in GV oocytes, MII, and PN zygotes but was not expressed in 2-cell embryos, 4–8-cell embryos, Mo, or BI (Fig. 7A). Western blot analysis showed that mouse cPLA2γ was expressed in GV stage oocytes and that a strong level of expression persists through the MII, PN, 2-cell, and 4–8-cell stages (Fig. 7B). By the morula stage, however, mouse cPLA2γ levels decrease dramatically and remain low

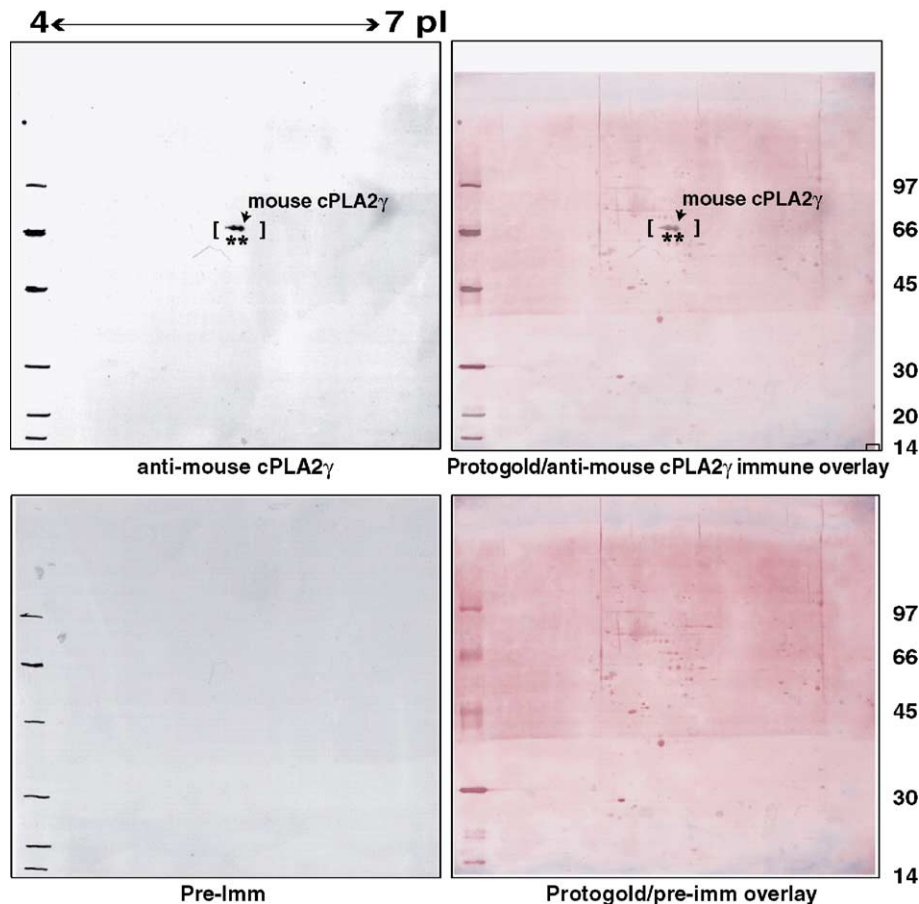


Fig. 5. Two-dimensional Western blot analysis demonstrating that mouse cPLA2 γ immune sera is reactive with the 67.8 kDa (pI 5.7) mouse cPLA2 γ protein spot. Mouse cPLA2 γ preimmune sera was not reactive with egg proteins. However, mouse cPLA2 γ immune sera strongly reacts with the protein spot which contained the mouse PLA2 γ peptides. An arrow indicates the spot which was originally cored for TMS analysis. Note that two other slightly more acidic proteins are reactive with the immune sera (asterisks), possibly indicating that mouse cPLA2 γ is post-translationally modified. Overlay image localizes reactive proteins with protein spots resolved using protogold.

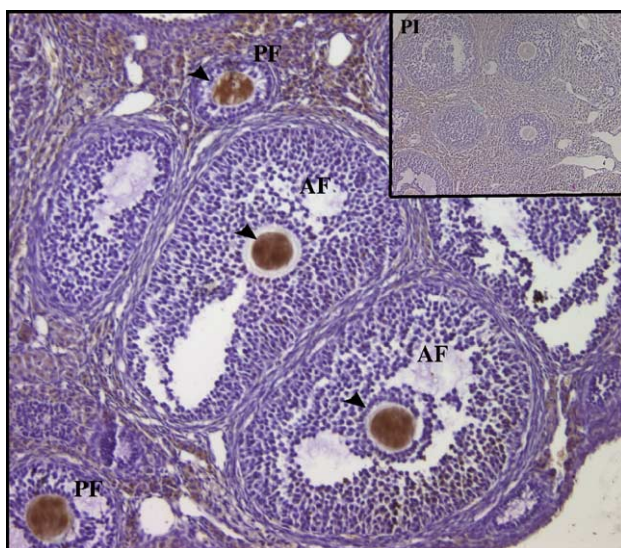


Fig. 6. Mouse cPLA2 γ localization in ovary. Ovarian cross-sections from mature females were probed with anti-mouse cPLA2 γ sera or preimmune sera (inset) and visualized using immunoperoxidase staining. Mouse cPLA2 γ expression was observed in oocytes (arrows) contained within preantral (PF) and antral follicles (AF). 100 \times . PI: preimmune sera.

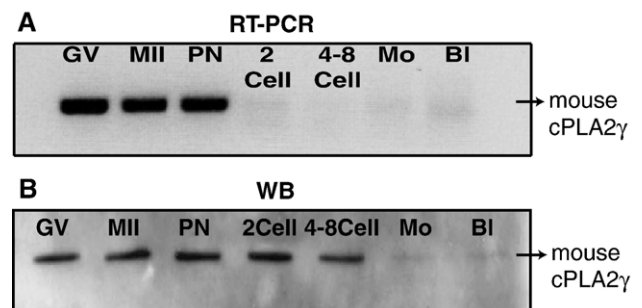


Fig. 7. Developmental expression of mouse cPLA2 γ in oocytes and embryos. Germinal vesicle (GV), metaphase II (MII), pronuclear (PN), 2-cell, 4–8-cell, morula (Mo), and blastocyst (BI) were collected from hormonally stimulated mice, and RNA was extracted for RT-PCR (A) or protein was extracted for Western blot analysis (B). (A) Specific primers based on the mouse cPLA2 γ open reading frame were used in PCR reactions. RNA transcripts could be visualized in GV staged oocytes, MII eggs, and PN staged embryos. (B) Western blots of oocyte/embryo extracts were probed with immune IgG. cPLA2 γ protein expression appeared highest in GV staged oocytes through 4–8-cell staged embryos and then declined at Mo and BI stages of development. Prior to immunostaining, the blots were probed with Ponceau to control for protein loading.

through the blastocyst stage. In order to determine the localization of mouse cPLA2 γ in oocytes and early embryos, immunofluorescent (IF) analyses were conducted using deconvolution microscopy. In support of our Western blot analysis, IF also showed that mouse cPLA2 γ was expressed predominantly in GV staged oocytes (Fig. 8B), zygotes (Fig. 8C) and during early embryonic cleavage divisions (Figs. 8D and E), with decreased staining observed in morula and blastocysts (Figs. 8F and G). At the subcellular level, mouse cPLA2 γ appears to mainly localize to the cortical region and nucleoplasm of oocytes and early stage blastomeres. Interestingly, in 2-cell and 4–8-cell embryos (Figs. 8E and F), blastomere cortical staining is mainly limited to regions that are in contact with the extracellular space. Preimmune serum was not reactive with GV stage oocytes (Fig. 8A), eggs, or preimplantation stage embryos. In addition, eggs stained with immune sera that had been preabsorbed with recombinant mouse PLA2 γ lacked both nuclear and cortical staining, indicating that the immune sera is specific for mouse cPLA2 γ and was generated against the original cored protein (Supplementary Fig. 3).

Dynamic targeting of mouse cPLA2 γ aggregates to the nuclear envelope during germinal vesicle breakdown

While investigating mouse cPLA2 γ localization in GV oocytes, we noticed that the protein sometimes appeared to concentrate in aggregates either in the oocyte cortex or around the nuclear envelope. We predicted that the different localization patterns may be due to targeting of the cPLA2 γ aggregates to the nuclear envelope during spontaneous germinal vesicle breakdown (GVBD). In order to test this hypothesis, we released pools of germinal vesicle stage oocytes from ovarian follicles, fixed the cultured oocytes at different time points, and stained them with mouse cPLA2 γ sera. Results showed that, at 0 to 40 min following the onset of spontaneous maturation, mouse cPLA2 γ aggregates were concentrated at two foci located near the oocyte cortex (Fig. 9B, arrows). At later time points (40 to 60 min), more abundant foci were then seen surrounding the nucleus, while the cortical cPLA2 γ foci were not observed (Fig. 9E, arrow). A search of the literature found that a unique γ -tubulin-positive egg-restricted structure termed multivesicular aggregates (MVA) (Calarco, 2000) has a localization pattern which was strikingly similar to that of the mouse cPLA2 γ aggregates. To investigate if cPLA2 γ might represent a component of the MVA complex, we then repeated the GVBD experiment and co-stained the oocytes with mouse cPLA2 γ (shown in green) and γ -tubulin (red staining, Fig. 9, upper panel). Results showed a strong co-localization of cPLA2 γ and γ -tubulin at the two cortical foci (time = 0) and at the nuclear aggregates (time = 40–60 min) (yellow staining, Figs. 9C and F, arrow). Our finding that mouse cPLA2 γ appeared to aggregate at the germinal vesicle ~40–60 min after the initiation of spontaneous maturation (when the nuclear envelope is known to begin to

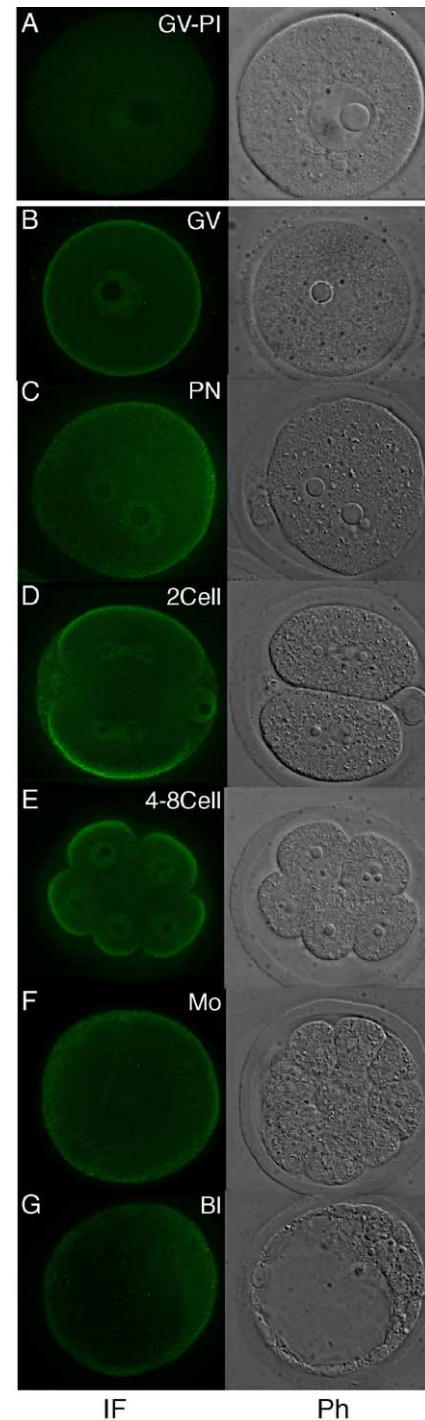


Fig. 8. Subcellular localization of cPLA2 γ in oocytes and preimplantation embryos. Germinal vesicle (GV) oocytes were obtained from ovaries of female mice. Metaphase II eggs (MII) were isolated from the oviducts of superovulated female mice. Pronuclear zygotes (PN), 2-cell, 4–8-cell, morula (Mo), and blastocyst (Bl) embryos were isolated from the oviducts and uterus of superovulated and mated female mice. All oocyte/embryos were fixed immediately after collection. Mouse cPLA2 γ protein was expressed in immature GV stage oocytes (B), PN (C), 2-cell (D), and 4–8-cell embryos (E). At the subcellular level, mouse cPLA2 γ mainly localized to the cortical region and the nucleoplasm. Protein levels were diminished in Mo and Bl staged embryos (F and G). The preimmune serum was not reactive with GV oocytes (A) or embryos (data not shown). IF—indirect immunofluorescence, Ph—phase contrast, 1000 \times .

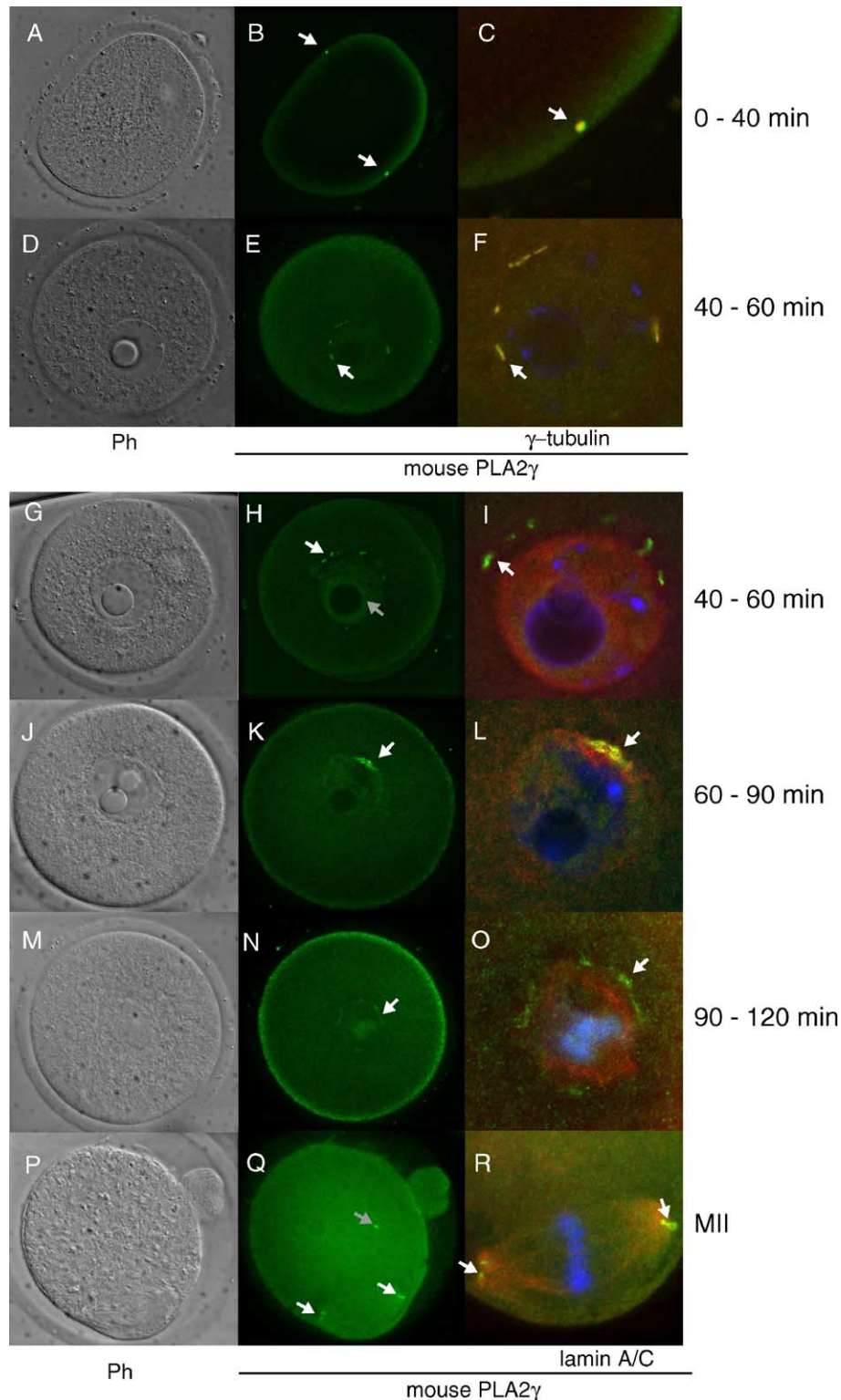


Fig. 9. Dynamic relocation of mouse cPLA2 γ aggregates from the oocyte cortex to the nuclear envelope during germinal vesicle breakdown. Oocytes were fixed at different time points (0, 40, 60, 90, 120 min, and 24 h) and co-stained with mouse cPLA2 γ immune sera (B, E, H, K, N, and Q) and either anti- γ -tubulin (C and F) or anti-lamin A/C (I, L, O, and R). For these images, mouse cPLA2 γ is shown in green, and γ -tubulin and lamin A/C are shown in red. Yellow staining indicates co-localization of cPLA2 γ with either γ -tubulin or lamin A/C. In GV oocytes fixed at time 0, two prominent cPLA2 γ aggregates were observed near the cortex (B, arrows). These aggregates also co-localized with γ -tubulin (C, arrow). At 40–60 min, the cPLA2 γ aggregates surrounded the nuclear envelope (E, arrow) and co-localized with γ -tubulin (F, arrow) but not lamin A/C (I, arrow). Between 60 to 90 min of culture, mouse cPLA2 γ concentrates at regions of the nuclear envelope which appeared ruffled and stain positive for lamin A/C (K and L, arrows). At 90–120 min culture, mouse cPLA2 γ aggregates were still found to surround lamin A/C and the condensed chromatin (N and O, arrow). At MII stage, mouse cPLA2 γ aggregates were found in the cytoplasm and concentrated at the spindle poles (Q and R). IF—immunofluorescence, Ph—phase contrast, 1000 \times .

undergo dissolution) suggested that mouse cPLA2 γ may be involved in this process. Therefore, we decided to perform more cPLA2 γ co-localization experiments using anti-lamin A/C antibodies (Ellenberg, 2002) in order to follow the association of cPLA2 γ with the nuclear envelope during GVBD (Figs. 9I, L, O, and R, shown in red). Results showed that, at 40 to 60 min, the mouse cPLA2 γ aggregates were found in accumulations surrounding the nuclear envelope (as noted above in Figs. 9H and I, white arrow). However, between 60 to 90 min, when the nuclear envelope takes on a more ruffled appearance (red staining, Fig. 9L, arrow), the mouse cPLA2 γ aggregates partially co-localize with lamin A/C suggesting a more intimate association with the nuclear membrane (Fig. 9L, arrow). Over the next 90 min, as the nucleus continued to undergo dissolution, the mouse cPLA2 γ aggregates remained in the cytoplasm closely apposed to lamin but were not seen to co-localize with lamin (Fig. 9O, arrow). In metaphase-II-arrested eggs, the mouse cPLA2 γ aggregates were found to be distributed throughout the cytoplasm (Fig. 9Q, gray arrow). Interestingly, positive staining was also observed at the poles of metaphase spindle (Figs. 9Q and R, arrows), perhaps suggesting a role for mouse cPLA2 γ in centrosomal function. When the nuclear envelope reformed at the pronuclear stage, strong cortical and homogeneous nuclear staining returned and persisted until the morula stage of development, while the mouse cPLA2 γ aggregates were no longer observed (Fig. 8C).

Discussion

In an effort to shed light on the molecular underpinnings of the mammalian oocyte, this report documents the characterization of a highly abundant novel egg and embryo-restricted maternal protein which we have named mouse cPLA2 γ . As a group, the heterogeneous PLA2 enzymes can be classified according to localization, sequence homology, and biochemical characteristics. Furthermore, they possess a diverse array of functions, including acylation regulation (Jackowski, 1996), membrane remodeling (Balsinde et al., 1997), cell proliferation (Roshak et al., 2000), and transcriptional regulation (Tashiro et al., 2004). The cytosolic (cPLA2) family consists of three isozymes, cPLA α (IVA), cPLA β (IVB), and the poorly characterized cPLA γ (IVC) (Kudo and Murakami, 2002).

Several characteristics of cPLA2 α include the protein sequence GLSGS, which is critical for cPLA2 α activity, a catalytic triad (serine 82, aspartate 385, arginine 54), and a regulatory calcium-dependent CaLB lipid binding domain. Underwood et al. (1998) originally cloned human cPLA2 γ and found that this novel family member contained a similar lipase consensus sequence (i.e. GVSGS) as well as the catalytic triad residues. However, human cPLA2 γ lacks the CaLB domain of cPLA2 α , suggesting that human cPLA2 γ may not require calcium for activity (Asai et al., 2003). The

investigators then utilized recombinant human cPLA2 γ to show that the protein preferentially catalyzes the hydrolysis of arachidonic acid at the *sn*-2 position of membrane phospholipids and is associated with cell membranes, likely via its myristoylation and prenylation motifs. Like human cPLA2 γ , mouse cPLA2 γ contains the lipase consensus sequence (GVSGS), the catalytic triad, and lacks the CaLB lipid binding domain. However, there are important differences between human and mouse cPLA2 γ . Unlike human cPLA2 γ , mouse cPLA2 γ lacks a prenylation signaling motif at its C-terminus (-CCLA) and a myristoylation signal sequence at its N-terminus [(MGXXX(S/small uncharged)-X)]. These findings suggest that perhaps mouse cPLA2 γ is not membrane anchored. This prediction is supported by our immunofluorescence analysis showing that a portion of the mouse cPLA2 γ appears to be distributed throughout both the cytoplasm and nucleoplasm. In several other cell types, PLA2s have been shown to translocate to either the nuclear envelope or nuclear fraction upon stimulation with calcium ionophore (Irvine, 2003), and therefore our findings that mouse cPLA2 γ partially localizes to the nucleus are not without precedent. Another important difference between human and mouse cPLA2 γ is that human cPLA2 γ is mainly limited to the heart and skeletal muscle, while mouse cPLA2 γ expression appears to be restricted to the oocyte and early embryo. This finding, coupled with the observation that mouse cPLA2 γ partially localizes to the nucleus and does not appear to be membrane anchored, suggests that human and mouse cPLA2 γ may have different biological functions.

With respect to the potential function of mouse cPLA2 γ , our immunofluorescence analysis shows that, when oocytes are isolated from follicles and fixed immediately, two large concentrated mouse cPLA2 γ foci are observed near the oocyte cortex. Following ~40 to 90 min of culture, however, the mouse cPLA2 γ foci are clustered around the nuclear envelope. This result suggests that the two cPLA2 γ foci migrate from the oocyte cortex towards the nuclear envelope during GVBD where they then fragment into smaller aggregates which surround the nucleus. We were struck by this finding and searched the literature to investigate if other molecules or structures showed similar dynamics during GVBD. Surprisingly, this staining pattern was highly reminiscent to that of a novel oocyte structure termed multivesicular aggregates (MVA). These large (~10 μ m) γ -tubulin-positive structures contain a variety of vesicular structures and are initially observed as two foci near the oocyte cortex but then break up into smaller fragments and surround the nuclear envelope during oocyte maturation. Some of these MVAs are then thought to eventually transform into the microtubule organizing centers (MTOC) following GVBD (Calarco, 2000; Palacios et al., 1993). Our finding that there is a precise co-localization of γ -tubulin and cPLA2 γ aggregates during GVBD strongly supports the hypothesis that cPLA2 γ represents a component of the novel MVA structure. Furthermore, the local-

ization of cPLA2 γ to the spindle poles in metaphase II-arrested eggs suggests that cPLA2 γ may also represent a component of the MTOC.

A signature feature of spontaneous GVBD is the nuclear envelop ruffling that occurs prior to membrane dissolution within a few hours of culture in vitro (Wassarman and Albertini, 1994). Although the mechanism by which GVBD occurs is unknown, in mitotic cells, nuclear envelope dissolution and reformation are known to be catalyzed by multiple enzymes, including phospholipases. We found that mouse cPLA2 γ often appeared to concentrate at the ruffled regions of the nuclear envelope during germinal vesicle breakdown (as determined by the co-localization of mouse cPLA2 γ and lamin shown in Fig. 9L) suggesting that this maternal protein may play a role in nuclear envelope dissolution during GVBD.

To conclude, mouse cPLA2 γ represents one of the more abundant proteins in the egg proteome. Northern blot and immunohistochemical analyses find that mouse cPLA2 γ expression is limited to the oocyte and early embryo. In the oocyte, mouse cPLA2 γ undergoes a striking migration from the oocyte cortex to the nuclear envelope during GVBD, suggesting a possible role in nuclear membrane remodeling. Furthermore, we show by Western blot and indirect immunofluorescence analysis that mouse cPLA2 γ protein continues to be expressed in embryos until the 4–8-cell stage of development suggesting a possible role for this maternal protein in early embryonic development. Therefore, we believe further consideration of the function of this novel phospholipase A2-like protein is warranted.

Acknowledgment

This work was supported by NIH grant HD38353.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2005.03.018](https://doi.org/10.1016/j.ydbio.2005.03.018).

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